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Analysis of tobacco-specific *N*-nitrosamines in indoor air

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A method was developed and applied for the assessment of tobacco-specific *N*-nitrosamines (TSNA) in indoor air polluted with tobacco smoke. Air samples were collected on Cambridge filters treated with 0.01 M potassium bisulfate, extracted with dichloromethane and enriched by column chromatography. The fraction containing the TSNA was concentrated and placed on a thermal desorption cartridge packed with Tenax GR. The sample was thermally desorbed and analyzed by capillary GC using a thermal energy analyzer. When the method was applied in a test laboratory in which one, two and four cigarettes were smoked during 30 min, linearity was observed. Field studies included sampling in bars, restaurants and trains. The concentration of *N'*-nitrosonornicotine (NNN) ranged from not detected to 23 pg/l, that of *N'*-nitrosoanatabine ranged from not detected to 9 pg/l, while 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was detected in concentrations ranging from 1 to 29 pg/l. This means an exposure to NNN and NNK of 0.1–0.3 cigarette equivalents. Thus, non-smokers can be exposed to highly carcinogenic TSNA.

Introduction

Epidemiological studies have incriminated involuntary smoking as a risk factor for lung cancer in non-smokers (1,2). Although the epidemiological data in respect to passive smoking and lung cancer are not fully substantiated, the IARC concluded that the material absorbed during passive smoking gives rise to some risk of cancer because of its physicochemical nature and in view of known concepts of chemical carcinogenesis (3).

Several tobacco-specific *N*-nitrosamines (TSNA*) have been identified in tobacco and tobacco smoke (4). These include *N'*-nitrosonornicotine (NNN), *N'*-nitrosoanatabine (NAT) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNN is formed by nitrosation from nicotine and nornicotine, NNK from nicotine and NAT from anatabine. Both NNN and NNK are powerful carcinogens that induce benign and malignant tumors of the lung, nasal cavity, esophagus, pancreas and/or liver in mice, rats and hamsters (5). Recently it was suggested that TSNA act as causative agents for lung cancer in active smokers (6). It was the goal of this study to assess the contribution of TSNA to indoor air pollution.

*Abbreviations: TSNA, tobacco-specific *N*-nitrosamines; NNN, *N'*-nitrosonornicotine; NAT, *N'*-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; TEA, thermal energy analyzer; DCM, dichloromethane; ETS, environmental tobacco smoke; VNA, volatile *N*-nitrosamines; NDMA, nitrosodimethylamine; NPYR, nitrosopyrrolidine

Materials and methods

Apparatus

A Model 543 thermal energy analyzer (TEA) (Thermo Electron Corp., Waltham, MA) was interfaced directly with a Model 5890 GC with a Model 3393A integrator and a 9114B disk drive (Hewlett Packard, Paramus, NJ). Thermal desorption was accomplished with a Chrompack Thermal Desorption Cold Trap Injector (no. 16230, Chrompack Inc., Raritan, NJ). A few modifications had to be made on the GC: the 70 W injection port heater was replaced with a 150 W heater (Chrompack no. 107960) and the back-pressure regulator was bypassed and replaced with a fore-pressure regulator (0–60 p.s.i.). Air sampling for TSNA was carried out using a portable vacuum pump Model Alpha 1 (Du Pont, Kennett Square, PA) while air samples for nicotine were taken with a Model S portable pump (Mine Safety Appliances Co., Pittsburgh, PA). Carbon monoxide was measured with a portable CO monitor (Model 211, National Draeger, Pittsburgh, PA). Calibration gas (60 p.p.m. CO in air) and flow controller were obtained by Catalyst Research Corp. (Baltimore, MD). Nicotine was analyzed on a Hewlett Packard Model 5890 GC equipped with an *N*-P detector and a Model 3392A integrator. For the generation of sidestream smoke, we used a 20-port smoking machine with a rotating head (H.Borgwaldt, Hamburg, Germany).

Materials

Reference cigarettes (2R1) were obtained from the Tobacco and Health Research Institute, University of Kentucky, Lexington, KY. Cambridge glassfiber filters (44 mm diameter) were obtained from the International Planters Corp., Richmond, VA (part no. R.20.0285.01) and Cambridge filter holders (type 1) were purchased from Filtrona Instruments and Automation Ltd (Milton Keynes, UK).

Reagents

NNN, NAT and NNK were synthesized according to earlier published methods (7–10). Nicotine and nornicotine standards were purchased from Roth-Atomergic Corp. (Farmingdale, NY) and quinoline and dibasic sodium phosphate were obtained from Sigma Chemical Co. (St Louis, MO). Potassium bisulfate (P-193) and citric acid (A-104) were acquired from Fisher Scientific (Springfield, NJ) while methanol, dichloromethane (DCM) and hexane were all HPLC grade and were purchased from J.T.Baker Inc. (Phillipsburg, NJ). Hydrobromic acid (no. 24,426–0) and ascorbic acid (no. A9,290–2) were obtained from Aldrich Chemical Co. (St Louis, MO) and glacial acetic acid was purchased from J.T.Baker, Inc. The 100 mg silica cartridge for sample clean-up (part no. 209000) and the Tenax GR (20–25 mesh) were obtained from Alltech Associates, Inc. (Deerfield, IL). Absorption traps (part no. 016250) were purchased from Chrompack Inc. (Raritan, NJ). Deactivated wide bore precolumn (0.53 mm i.d., part no. 160–2535) and the analytical GC column (30 m×0.32 mm i.d. DB-5, 0.25 μm film thickness, part no. 123–5032) were purchased from J&W Scientific (Folson, CA). The GB-5 column used for the analysis of tobacco alkaloids was obtained from On-Site Instruments (Columbus, OH).

Analysis of TSNA in indoor air

Initially, we analyzed the air of a smoke-filled test laboratory (16 m^3) under controlled conditions during which time one, two and four Kentucky 2R1 reference cigarettes per 30 min were machine smoked under standard smoking conditions (35 ml puff volume, 2 s puff duration and one puff/min; 11). Ventilation was only minimal. Air samples were collected on a Cambridge filter treated with 0.01 M potassium bisulfate solution (12) using a portable vacuum pump (Du Pont Alpha 1). Air (135 l) was collected through the Cambridge filter at a flow rate of 1.5 l/min. The Cambridge filter was extracted for 1 h by shaking with DCM. The extract was then concentrated to 0.5 ml and chromatographed on a 100 mg silica cartridge using DCM (2 ml), 2% methanol in DCM (3 ml) and finally methanol (2 ml). Usually the entire TSNA fraction was present in the DCM fraction containing 2% methanol. The TSNA fraction was dried, redissolved in 25 μl methanol and placed on a thermal desorption cartridge packed with 200 mg Tenax GR. The TSNA were desorbed at 220°C for 12 min (He flow rate: 10 ml/min) and cryofocused onto a 30 cm deactivated precolumn (0.53 mm i.d.) at 0°C. Prior to GC-TEA analysis, the cryotrap was ballistically heated from 0 to 220°C at 15°C/s. The GC oven was held at 40°C for 2 min and then ramped to 220°C at 6°C/min where it was held for 10 min. Under these conditions, the retention times of NNN, NAT and NNK were 25.4, 26.3 and 28.8 min respectively (Figure 1). Finally the GC oven was programmed to 275°C at 25°C/min and

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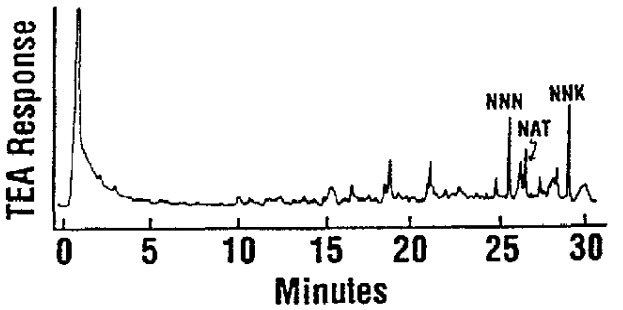

Fig. 1. Gas chromatogram of TSNA found in indoor air (bar II, 576 l).

held for 1 min (this clears residual compounds from the column). Using this method with standards only (external standard method), the recoveries of NNN, NAT and NNK in the range 1–50 pg/l were 36 ± 1.0, 36 ± 0.8, and 56 ± 2.8% respectively (despite efforts in changing the various parameters, we were unable to secure higher reproducible recovery rates). No breakthrough of TSNA was observed when the treated Cambridge filters were used. The detection limit of this method is ~0.1–0.5 ng per total sample, depending on chromatographic optimization. In some samples we observed some chromatographic interferences at the retention time of NNN and NAT. Field samples were taken at several locations, such as bars, restaurants and trains at flow rates ranging from 2.1 to 3.3 l/min (total sample size collected varied from 576 to 1287 l).

Confirmatory methods

Since the mere TEA response at trace levels does not necessarily prove the identity of a compound, we applied confirmatory microreactions, such as UV irradiation at 365 nm and treatment with 10% hydrogen bromide in glacial acetic acid (13). The TSNA peaks were confirmed to be *N*-nitrosamines.

Monitoring of artefact formation

When *N*-nitrosamines are analyzed at trace levels, it is important to take any possible precaution that they are not artefactually formed during sampling and/or analytical work-up (14). In order to assure that TSNA were not formed artefactually, we analyzed their precursor amines nicotine, nornicotine and anatabine in the test laboratory under extreme conditions (four cigarettes smoked per 30 min for a total of 90 min). During that time 135 l of air were collected on a treated Cambridge filter at 1.5 l/min. We detected 36 μg nicotine and 3 μg nornicotine total; no anatabine was detected. These levels are considered much higher than those expected in field samples. We then applied 50 μg of nicotine and 3.7 μg of nornicotine onto the thermal desorption tube and applied the analytical conditions described before. No TSNA peaks were observed. We also spiked a treated Cambridge filter with 50 μg of nicotine and 3.7 μg of nornicotine. We then sampled 135 l of tobacco smoke-polluted air from the test laboratory under extreme conditions through the spiked Cambridge filter while an identical air sample was drawn through an unspiked treated Cambridge filter under the same conditions. There was no increase in the TSNA levels of the spiked filter pad compared to the unspiked one. Additionally, we employed two midget impingers filled with 10 ml each of citrate–phosphate buffer, pH 4.5, containing 20 mM ascorbic acid (15). A 135 l air sample (under extreme smoking conditions) was drawn through the two impingers before being collected on a Cambridge filter while a parallel sample was collected simultaneously without allowing it to pass through the scrubber solution. The sample that passed through the scrubber solution had slightly less NNN and NAT due to absorption of the compounds in the aqueous solution (an attempt to recover the difference by extracting the buffer with DCM was unsuccessful because of the low levels), while the levels of NNK were identical (351 pg/l in the scrubber sample and 349 pg/l in the sample collected directly on the Cambridge filter). This, in our judgement, demonstrates that there was no artefact formation.

Determination of nicotine

Under the most extreme conditions in the test laboratory, we collected 135 l air onto a treated Cambridge filter. The filter was extracted for 2 h by shaking with 5 ml of 1% (w/v) KOH in methanol containing 64.5 μg ml quinoline as internal standard. A 2 μl aliquot was analyzed by GC using a 50-m WCOT fused silica capillary column with spitless injector. The injection port temperature of 200°C and the detector temperature of 230°C were maintained. The GC oven was programmed from 50 to 110°C at 30°C min, with an initial 1 min hold, it was then kept at 110°C for 3 min before being raised to 210°C at 6°C min where it was held for 10 min.

Table I. Tobacco-specific *N*-nitrosamines in indoor air (pg/l)

Parameters		NNN	NAT	NNK
1 cigarette per	run no. 1	8.6	3.6	107
half hour	run no. 2	11.2	5.4	135
(3 cig. total)	average	10.0	4.5	121
2 cigarettes per	run no. 1	15.3	12.3	207
half hour	run no. 2	20.6	14.4	188
(6 cig. total)	average	17.9	13.4	198
4 cigarettes per	run no. 1	42.7	93.5	448
half hour	run no. 2	51.3	111	493
(12 cig. total)	average	47.0	102	471

Test lab 16 m^3, limited ventilation. Cigarettes were machine smoked. Air samples were collected on Cambridge filters (44 mm, treated with 0.01 M $KHSO_4$) at a flow rate of 1.5 ml/min for 90 min (135 l total).

Table II. Tobacco-specific *N*-nitrosamines in indoor air (pg/l)[a]

Site	Approx. no. of cigarettes smoked	Collection time (h)	Flow rate (l/min)	TSNA		
				NNN	NAT	NNK
Bar I	25–35	3	3.2	22.8	9.2	23.8
Bar II	10–15	3	3.2	8.3	6.2	9.6
Bar III	10–15	3	3.2	4.3	3.7	11.3
Restaurant I[b]	25–30	6	2.15	1.8	1.5	1.4
Restaurant II[b]	40–50	8	2.1	ND	ND	3.3
Car[c]	13	3.3	2.15	5.7	9.5	29.3
Train I	50–60	5.5	3.3	ND	ND	4.9
Train II	50–60	6	3.3	ND	ND	5.2
Office	25	6.5	3.3	ND	ND	26.1
Smoker's home	30	3.5	3.3	ND	ND	1.9

[a]Data corrected for recovery.
[b]Smoking section.
[c]Windows partially open.
ND, not detected (in some cases due to chromatographic interference).

Table III. Comparison of *N*-nitrosamines in indoor air (pg/l)[a]

Site	VNA		Site	TSNA	
	NDMA	NPYR[b]		NNN	NNK
Train (bar)	130	ND	Train	ND	5
Office	2–66	0–36	Office	0.7–6	0.2–10.7
Coffee shop	12–27	19–21	Restaurant	2	1–3
Sports hall	90	ND	Car	6	29
Dance hall	70	ND	Bar	4–23	11–24

NDMA, nitrosodimethylamine; NPYR, *N*-nitrosopyrrolidine; ND, not detected.
[a]From refs. 19–22.

Determination of carbon monoxide

It was thought that the levels of CO could be an indicator for the degree of smoke pollution. A portable CO monitor was used in the test laboratory as well as at some field sites. It was equipped with a CO-specific interference diffusion filter.

Results and discussion

Figure 1 shows a representative chromatogram of the TSNA fraction from a tobacco smoke-polluted indoor environment (bar II; Table II). In some cases, some chromatographic interferences at the retention time of 26 min prevented the assessment of NNN

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and NAT due to integration errors. The thermal desorption unit was used because it offers the advantage that the entire sample can be analyzed rather than only an aliquot. During the method development we also studied the effect of ageing of the samples. When two identical air samples were collected on treated Cambridge filter pads in the test laboratory and analyzed 3 days apart, the TSNA levels were identical, indicating good stability for that time span. This may be important since sometimes field samples cannot be processed immediately.

Table I shows the TSNA levels obtained under different conditions in the test laboratory in which one, two and four cigarettes per 30 min were machine smoked simultaneously. During a 90 min span, 135 l of air was drawn through treated Cambridge filters at 1.5 l/min. Each sample was analyzed in duplicate. There is a reasonably good dose linearity for both NNN and NNK but not for NAT. It is noteworthy that the NNK levels are ~10 times higher than those of NNN. In cigarette smoke, NNK has about twice the level in sidestream smoke than in mainstream smoke (9,16). Also, the sidestream to mainstream ratio of NNK is ~10 times larger than that of NNN (16). Table II lists the TSNA values obtained at different indoor sites polluted with environmental tobacco smoke (ETS). The number of cigarettes smoked during the sampling time (3–8 h) was estimated to the best of our ability. NNN levels varied from not detected to 22.8 pg/l while NAT ranged from not detected to 9.5 pg/l. NNK levels ranged from 1.4 to 29.3 pg/l. (In field samples, the ratio of NNK to NNN was not as pronounced as it was in the small test laboratory, possibly because of the much larger size and different surroundings of these sites.) Assuming a respiratory rate of 10 l/min, a non-smoker exposed to these concentrations of TSNA may inhale up to 41 ng of NNN and up to 43 ng of NNK in 3 h. Taking as an average value of NNN and NNK in the mainstream smoke of a filter cigarette 360 and 130 ng/cigarette respectively, this would mean an exposure to NNN of 0.1 and to NNK of 0.3 cigarette equivalents (9,16). According to the Surgeon General's report, a non-smoker can be exposed to up to 0.09 mg nicotine in 3 h which corresponds to the nicotine content of ~0.1 cigarette (1).

The levels of volatile *N*-nitrosamines (VNA) in sidestream smoke are at least 10 times (and up to 50 times) higher than in mainstream smoke (17,18). Several studies have dealt with the measurement of VNA in ETS (19–22). Table III presents a comparison of the levels of VNA and TSNA. In general, values of VNA exceed those of TSNA by ~5–10 times, though some are in the same order of magnitude.

In an earlier study, Klus *et al.* measured several ETS-related compounds including NNN and NNK in indoor air of an office (83 m^3) under various smoking conditions (22,23). While NNN levels varied from 0.7 to 6 pg/l, NNK was detected at concentrations of 0.2–10.7 pg/l. These findings are in line with our data (Table II). Klus *et al.* also measured CO levels during that time, detecting from 3 to 14 p.p.m., without any apparent correlation to the TSNA concentrations. In our hands, CO concentrations rose up to 15 p.p.m. when four cigarettes were smoked simultaneously in the test laboratory. When one cigarette was smoked every 10 min, the CO concentration stabilized around 6 p.p.m. In some field samples, CO levels ranged from 0.5 (office) to 2 p.p.m. (restaurant). We concluded that CO concentrations alone are not an indicator for the amounts of TSNA present in ETS. In conclusion, we have shown that the carcinogenic NNN and NNK can be assessed in ETS and were found at levels up to 23 pg NNN/l and 29 pg NNK/l (ng/m^3). Thus it is possible to estimate the amounts of TSNA a non-smoker may be exposed to when residing in tobacco smoke-polluted environments.

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